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Highly specific and sensitive liquid chromatography–tandem mass spectrometry method for the determination of 3-desmethylthiocolchicine in human plasma as analyte for the assessment of bioequivalence after oral administration of thiocolchicoside

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Abstract

A sensitive method for the determination of 3-desmethylthiocolchicine in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The plasma samples were extracted with ethyl acetate and separated on a Phenomenex Luna $C_{18}(2)$ 5 μm, 150×2 mm column with a mobile phase consisting of acetonitrile–0.005% formic acid (350:650, v/v) at a flow rate of 0.35 ml/min. Detection was achieved by an Applied Biosystems API 2000 mass spectrometer (LC–MS–MS) set at unit resolution in the multiple reaction monitoring mode. TurboIonSpray ionisation was used for ion production. The mean recovery for 3-desmethylthiocolchicine was 70%, with a lower limit of quantification set at 0.39 ng/ml. The increased selectivity of mass spectrometric (MS–MS) detection allowed us to distinguish between thiocolchicoside and its primary metabolite 3-desmethylthiocolchicine in human plasma, thereby giving more insight about the pharmacokinetics of the drug in humans.

Keywords: Thiocolchicoside; 3-Desmethylthiocolchicine; Organosulfur compounds; Colchicine

1. Introduction

Thiocolchicoside, is a synthetic sulphur derivative of colchicoside, a naturally occurring glucoside contained in the *Colchicum autumnale* plant [1]. Thiocolchicoside has a selective affinity for γ-aminobutyric acid (GABA) receptors and acts on the muscular contracture by activating the GABA-nergic inhibitory pathways thereby acting as a potent muscle relaxant [1,2].

According to literature, thiocolchicoside is absorbed rapidly from the gastrointestinal tract, after oral administration and peak plasma concentrations are observed within approximately 1 h [1]. In pilot studies conducted in three normal volunteers given a single 4.26 mg oral dose of tritium-labelled drug, plasma radioactivity was found to reach a peak after 0.5–1 h and to be eliminated with a terminal half-life of about 8 h [1]. A meaningful interpretation of these data was complicated by the fact that the parent compound was not differentiated from possible metabolites [1].

Analytical methods published for the determination of thiocolchicoside to date, are either non-spe-

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cific radioimmunoassay techniques [3,4], or use enzymatic hydrolysis of the thiocolchicoside to the 3-desmethylthiocolchine (aglycone) product [5]. Perruca et al. [5] described a gas chromatography–mass spectrometry (GC–MS) method for the determination of thiocolchicoside after enzymatic hydrolysis to its aglycone (3-desmethylthiocolchicine) and no attempt was made to account for the possible occurrence of deconjugation in vivo. This could lead to a serious over estimation of the ''thiocolchicoside'' plasma levels. Sandouk et al. [4] used a radioimmunoassay method using cross-reacting colchicine-specific polyclonal antibodies and [^{3}H]colchicine as marker. Cross-reactivity was 56% for thiocolchicoside and 100% for colchicine. Cross-reactivity tests were performed for some colchicine analogues, but not for 3-desmethylthiocolchicine (aglycone of thiocolchicoside). Cross-reactivity with the aglycone is therefore highly likely. The huge differences between the C_{max} values after oral administration of an 8-mg dose of the same formulation, as reported by Perucca et al. [5] (18.7 ng/ml) and Sandouk et al. [3] (104 ng/ml), can probably be ascribed to non-specificity of the radioimmunoassay methods used by the latter investigators.

We therefore decided to develop a highly specific and sensitive liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the determination of thiocolchicoside in plasma using solid-phase extraction. It was, however, discovered that thiocolchicoside is metabolised so rapidly after oral administration that it is impractical to determine concentrations in plasma over the periods generally used for bioequivalence studies. Further investigation revealed that the aglycone, 3-desmethylthiocolchicine is the major metabolite and appears in plasma at concentrations which can be assayed over the required periods and therefore represents an appropriate surrogate analyte for the assessment of bioequivalence after oral administration of thiocolchicoside.

This report describes a highly sensitive and specific LC–MS–MS method for the determination of 3-desmethylthiocolchicine in human plasma with a simple liquid–liquid extraction procedure and a total chromatography time of 3.0 min. The method used to determine thiocolchicoside will also be discussed although it was not validated.

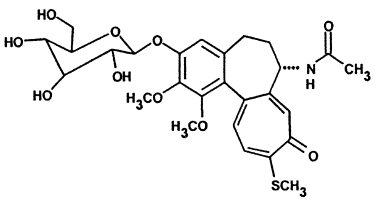
2. Experimental

2.1. Materials and chemicals

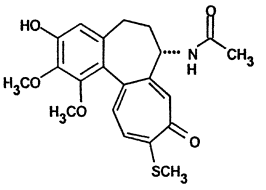
A Phenomenex Luna C_{18}(2) 5 μm, 150×2.1 mm column (Phenomenex, Torrance, CA, USA) was used for separation at a flow-rate of 0.35 ml/min and injecting 30 μl onto the column (for both thiocolchicoside and 3-desmethylthiocolchicine). The mobile phase was delivered by a Perkin-Elmer Series 200 micropump and the samples were injected by a Perkin-Elmer Series 200 autosampler. Detection was performed by an Applied Biosystems API-2000 detector (Applied Biosystems, Canada) using Turbo-IonSpray ionisation (electrospray ionisation, ESI) for ion production.

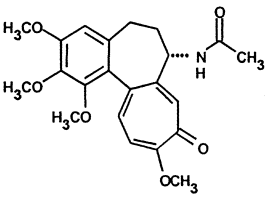
Tris buffer (0.5 *M*) was prepared and adjusted to pH 8.0 with hydrochloric acid (32%).

Thiocolchicoside, $C_{27}H_{33}NO_{10}S$, and 3-desmethylthiocolchicine, $C_{21}H_{23}NO_5S$ were supplied by Galenix Development, France and Bioflore, France, respectively. Colchicine, $C_{22}H_{25}NO_6$, internal standard was taken from the Farmovs-Parexel Clinical Research Organisation internal pure substance reference material library.



Thiocolchicoside





3-desmethylthiocolchicine Colchicine

2.2. Extraction procedure

A 3-desmethylthiocolchicine standard stock solu-

tion was made up in methanol and used immediately to spike plasma and discarded thereafter. Calibration standards and quality control standards were prepared in normal human plasma by spiking a pool of normal plasma which was then serially diluted with normal blank plasma to obtain a series of eight calibration standards spanning a concentration range of 0.39–50.4 ng/ml. The calibration standards and quality control standards were aliquoted into tubes and stored under the same conditions as the trial samples at −20 °C. Calibration standards for thiocolchicoside (0.30–37.6 ng/ml) were prepared in a similar fashion and stored at −20 °C.

2.2.1. 3-Desmethylthiocolchicine

To 1.0 ml plasma containing 3-desmethylthiocolchicine in a 10-ml amber glass ampoule was added 50 μl colchicine internal standard solution (672 ng/ml in water), 200 μl 0.5 *M* Tris buffer (pH 8.0) and 3 ml ethyl acetate. The sample was vortex mixed for 1.5 min and centrifuged at 1300 *g* for 1 min at 8 °C.

The aqueous phase was frozen at −30 °C on a Fryka Polar cooling plate (Kältetechnik, Esslingen, Germany) and the organic phase decanted into a clean 5 ml amber glass ampoule. The organic phase was evaporated under vacuum on a Savant SpeedVac (Savant Instruments, NY, USA) evaporator at ambient temperature. The extract was reconstituted with 200 μl mobile phase, transferred to an autosampler vial insert and 30 μl injected onto the HPLC column.

2.2.2. Thiocolchicoside

Extraction of the plasma samples containing thiocolchicoside, was performed using 100 mg HCX solid-phase extraction columns (International Sorbent Technology).

To 1.0 ml plasma in a 5-ml polypropylene tube was added 1 ml ammonium acetate (0.05 *M*, pH 6.0) and the sample briefly vortexed. The solid-phase extraction (SPE) columns were activated by passing 1 ml of methanol, followed by 1 ml of ammonium acetate (0.05 *M*, pH 6.0) through the columns under vacuum. The plasma mixture (2 ml) was passed through the column by positive pressure and the column washed with 1 ml of ammonium acetate (0.05 *M*, pH 6.0). The analyte was eluted with 1 ml of acetonitrile–ammonium hydroxide (5 *M*) (95:5, v/v) and the samples evaporated to dryness using a Savant SpeedVac concentrator at ambient temperature. The extract was reconstituted with 200 μl mobile phase, transferred to an autosampler vial insert and 30 μl injected onto the HPLC column.

2.3. Liquid chromatography

2.3.1. 3-Desmethylthiocolchicine

Chromatography was carried out at ambient temperature with a mobile phase consisting of acetonitrile–0.005% formic acid (350:650, v/v) at a flow-rate of 0.35 ml/min.

2.3.2. Thiocolchicoside

Chromatography was carried out at ambient temperature with a mobile phase consisting of acetonitrile–0.05% formic acid (250:750, v/v) at a flow-rate of 0.35 ml/min.

2.4. Mass spectrometry

Electrospray ionisation was performed in the positive ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 70, 75 and 55 (respective arbitrary values). The ESI temperature was set at 400 °C. The pause time was set at 5 ms and the dwell time at 200 ms.

The Applied Biosystems API 2000 LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions m/z 402.0, 564.0 and 400.0 to the product ions m/z 360.2, 402.0 and 358.0 (for 3-desmethylthiocolchicine, thiocolchicoside and colchicine, respectively). Fig. 1 shows the single parent (m/z 402.0) to product ions MS–MS of 3-desmethylthiocolchicine, Fig. 2 shows the single parent (m/z 564.0) to product ions MS–MS of thiocolchicoside and Fig. 3 shows the single parent (m/z 400.0) to product ions MS–MS of colchicine. The collision energies for 3-desmethylthiocolchicine, thiocolchicoside and colchicine were 27, 29 and 25 eV, respectively.

ESI was used for ion production and the collision gas (N_2) set at 3 (arbitrary value).

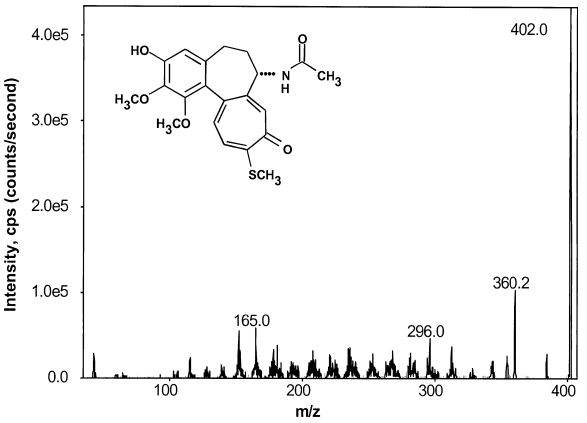


Fig. 1. Full mass spectrum of the protonated 3-desmethylthiocolchicine molecular ion (m/z 402.0, molecular structure given) and the principal product ions formed after collision (MS–MS).

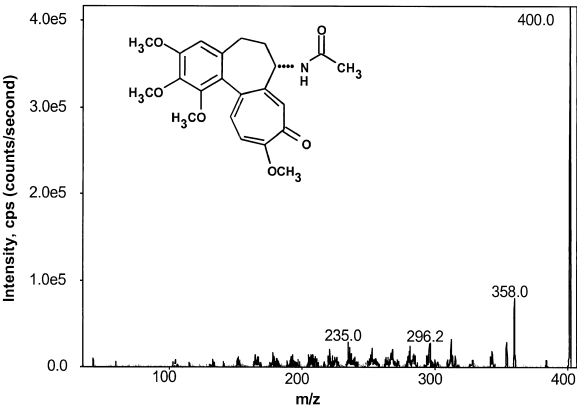


Fig. 3. Full mass spectrum of the protonated colchicine molecular ion (m/z 400.0, molecular structure given) and the principal product ions formed after collision (MS–MS).

2.5. Validation (3-desmethylthiocolchicine)

The method was validated for 3-desmethylthiocolchicine by analysing plasma quality control samples six times at seven different concentrations, i.e. 45.2, 22.6, 11.3, 3.80, 1.90, 0.95 and 0.48 ng/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing eight different concentrations spanning the concentration range 50.4–0.39 ng/ml. Calibration graphs were constructed using a weighted linear regression (1/concentration) of the drug/internal standard peak-area ratios of the product ions for the analyte and the internal standard, versus the nominal drug concentrations. The weighted linear regression (1/concentration) was found to be the simplest regression, giving the best results for both analytes.

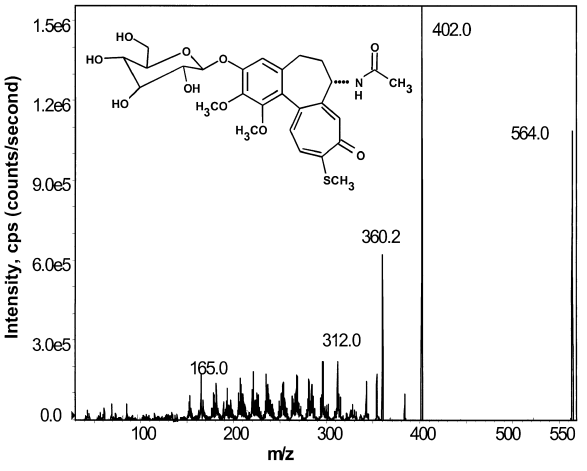


Fig. 2. Full mass spectrum of the protonated thiocolchicoside molecular ion (m/z 564.0, molecular structure given) and the principal product ions formed after collision (MS–MS).

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated by extracting ''blank'' biological fluids from 10 different sources, reconstituting the final extract in injecting solvent containing a known amount of the analyte, analysing the reconstituted extracts and then comparing the peak areas of the analytes. Absolute recoveries of the analyte and metabolite were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with 3-desmethylthiocolchicine. Recoveries were calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted system performance verification standard representing 100% recovery. The system performance verification standard was prepared by spiking ''blank'' extracts with known amounts of the analyte and internal standard.

2.6. ''Specificity samples''

To establish the appropriate calibration range for

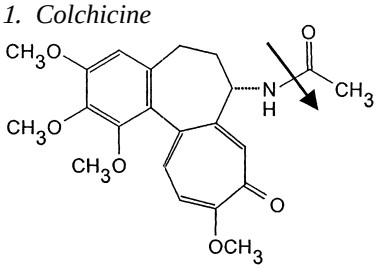
the analysis of a drug in plasma, all the plasma remaining after sufficient aliquots had been taken for pharmacokinetic analysis, are combined for each time-point of blood collection (''specificity samples''). These samples are then analysed together with standards (similar concentrations as reported in the literature) using the extraction and detection method described for the analyte of interest, in order to prepare appropriate ranges of the standards and quality controls to be used in the validation of the assay method and during the assaying of the study samples.

3. Results and discussion

Initially a method was developed to determine thiocolchicoside in plasma. The response was linear over the range 0.30–37.6 ng/ml in extracted plasma calibration standards (r^2=0.997). This indicated a suitable method sufficiently sensitive to determine unchanged thiocolchicoside in plasma for up to five half-lives based on the C_{max} of 18.7 ng/ml reported by Perruca et al. [5]. The mean absolute recovery of thiocolchicoside determined in triplicate at 15.2 ng/ml was 75% (RSD 3.1%) and with a retention time of 2.02 min, a total chromatography run time of 2.5 min was allowed. After the method was developed and the lower limit of quantification (LLOQ) determined (0.30 ng/ml) the ''specificity samples'' were analysed together with plasma calibration standards in order to prepare appropriate ranges of the standards and quality controls to be used in the validation of the assay method and during the assaying of the study samples. The results showed that only minute traces of thiocolchicoside were present in the specificity plasma samples. The concentrations were all below the LLOQ (<0.30 ng/ml) apart from the first sampling point (30 min after dosing) which was approximately equal to the LLOQ concentration. Since the methods described in the literature were all non-specific methods, these results lead to the conclusion that the ''thiocolchicoside'' levels measured in these publications were probably due to other analogues, most likely the aglycone (3-desmethylthiocolchicine) which occurs after deconjugation of thiocolchicoside. This was verified by a chromatographic peak found at 6.75 min after re-injection of the ''specificity samples'' and allowing 20 min chromatography time, while monitoring for the aglycone (M+1, m/z 402.0).

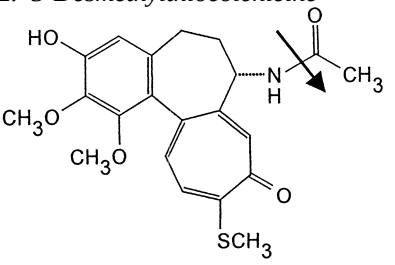
To further elucidate the structure of the component eluting at 6.75 min, a solution of colchicine, an analogue of 3-desmethylthiocolchicine, was infused into the mass spectrometer and the system tuned for optimum product ion formation (MS–MS) (Fig. 3). The product ion of highest abundance formed from the molecular ion of m/z 400.0 was m/z 358.0, suggesting that it was a loss of an acetyl group forming *N*-desacetylcolchicine (m/z 358.0).

3.1. Colchicine



Since the 3-desmethylthiocolchicine had basically the same structure as colchicine it suggested that in the MS–MS mode the 3-desmethylthiocolchicine would also be de-acetylated to form *N*-desacetyl–3-desmethylthiocolchicine with a m/z=360.

3.2. 3-Desmethylthiocolchicine



In the next experiment, the MS–MS conditions were kept the same as for the colchicine, but the transition of the molecular mass (m/z 402.0) to the predicted *N*-desacetylated product ion (m/z 360.0) was monitored for 3-desmethylthiocolchicine. In all the specificity plasma samples significant amounts of 3-desmethylthiocolchicine could be found. A pure reference standard of 3-desmethylthiocolchicine was therefore obtained and the assay method developed to determine the aglycone metabolite as analyte for the assessment of thiocolchicoside bioequivalence.

Table 1
Intra-day quality control results for 3-desmethylthiocolchicine

Nominal concentration (ng/ml)	Mean concentration found (ng/ml) (n=6)	RSD (%)	% Nominal
45.2	44.3	4.4	98
22.6	22.1	4.9	98
11.3	11.5	5.0	102
3.80	3.77	6.2	99
1.90	1.91	6.2	101
0.95	0.97	6.5	102
0.48	0.49	6.7	102

The mean absolute recovery of 3-desmethylthiocolchicine determined in triplicate at 45.2, 11.3 and 1.90 ng/ml was 66 (RSD 3.8%), 70 (RSD 2.1%) and 67% (RSD 4.1%), respectively, while for colchicine (internal standard), the mean recovery at 33.0 ng/ml was 60% (RSD 4.1%). No matrix effect for 3-desmethylthiocolchicine and colchicine was observed for 10 different plasma pools.

The LLOQ is defined as that concentration of 3-desmethylthiocolchicine which can still be determined with acceptable precision (RSD<20%) and accuracy (bias<20%) and was found to be 0.39 ng/ml. Results from the intra-day validation assays indicate a valid calibration range of 0.39–50.4 ng/ml (r^2=0.998). The intra- and inter-day assay method performance statistics are presented in Tables 1 and 2.

On-instrument stability was inferred from special stability samples which were prepared and included in the validation batch. No significant degradation could be detected in the cooled samples (4 °C) left on the autosampler for at least 28 h.

Colchicine was found to be a suitable internal standard as it had an acceptable recovery (60%) and eluted close to the analyte of interest thereby minimising any potential matrix effects.

Retention times were 2.32 and 2.03 min for 3-desmethylthiocolchicine and colchicine, respectively. A total chromatography run time of 3.0 min was allowed, which made it possible to analyse large batches of samples per day. Fig. 4 shows representative chromatograms obtained for 3-desmethylthiocolchicine at 0.39 ng/ml (LLOQ) and for a study sample close to the limit of quantification at the late elimination phase of the pharmacokinetic profile.

The method was employed to analyse plasma samples containing 3-desmethylthiocolchicine obtained after a single oral dose of 8 mg thiocolchicoside per treatment phase in 44 healthy volunteers. Concentration versus time profiles were constructed for up to 24 h (Fig. 5). The maximum 3-desmethylthiocolchicine plasma concentrations obtained varied between 4.85 and 26.3 ng/ml. The elimination half-life of 3-desmethylthiocolchicine was found to be 0.8 h. An interesting observation is the increase in the 3-desmethylthiocolchicine plasma concentrations found in 43 of the 44 subjects during the elimination phase of the pharmacokinetic profile at approximately 6 h. This may be due to an intrahepatic cycle of the drug, but was not investigated further.

In these methods we made use of the increased sensitivity and selectivity of MS–MS detection to

Table 2
Inter-day quality control results for 3-desmethylthiocolchicine

	Nominal (ng/ml)				
	0.48	0.95	1.90	11.3	22.6
Mean	0.48	0.95	1.92	11.4	22.6
RSD	6.7	5.6	4.9	3.7	4.3
% Nominal	100	100	101	101	100
n	18	18	18	18	18

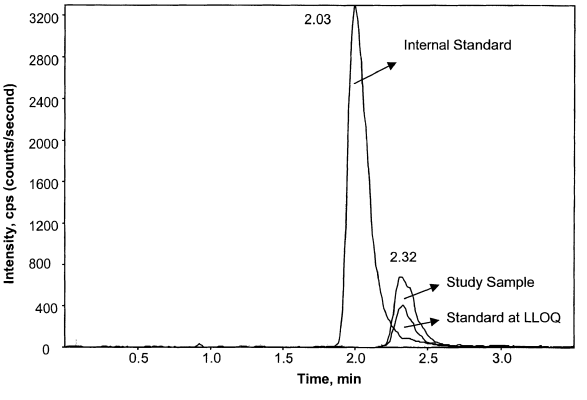


Fig. 4. High-performance liquid chromatograms of the calibration standard at the lower limit of quantification (LLOQ) containing 0.39 ng/ml 3-desmethylthiocolchicine and of a study sample at the late elimination phase of the pharmacokinetic profile of the analyte containing 0.71 ng/ml 3-desmethylthiocolchicine.

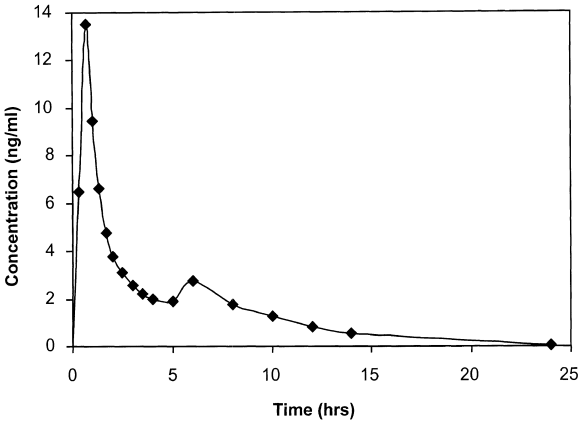


Fig. 5. Representative 3-desmethylthiocolchicine mean plasma concentrations versus time profile as obtained after a single 8-mg oral dose of thiocolchicoside (44 subjects, two phases).

develop highly specific and accurate methods for the determination of thiocolchicoside and its major metabolite 3-desmethylthiocolchicine in plasma thereby enabling us to gain new information about the pharmacokinetics of thiocolchicoside in the human body.

4. Conclusion

A highly specific, accurate and sensitive method was developed for the determination of thiocolchicoside in plasma samples, using liquid chromatographic separation and mass spectrometric detection (LC–MS–MS). This method was applied in the analyses of specificity plasma samples generated in a pharmacokinetic study in order to ascertain the mean plasma concentrations of thiocolchicoside after an oral dose of 8 mg to human volunteers. Only very low concentrations of the drug could be detected in these samples (below 0.30 ng/ml).

These results suggest that thiocolchicoside is rapidly converted to 3-desmethylthiocolchicine (possibly partially in the acidic stomach juices) during absorption and during the first-pass effect through the liver. This indicates that the literature describing the pharmacokinetic data of thiocolchicoside is incorrect, probably reporting the combined concentrations of small amounts of the thiocolchicoside left in the plasma together with the aglycone 3-desmethylthiocolchicine as the main component. It is therefore recommended that in order to perform bioequivalence studies on thiocolchicoside, the plasma samples should be analysed for 3-desmethylthiocolchicine concentrations and not for thiocholchicoside.

A highly specific and sensitive method for the determination of 3-desmethylthiocolchicine in plasma was developed and all the plasma samples generated during the study were analysed using this method allowing us to determine plasma concentrations up to 24 h after a single oral dose of 8 mg thiocolchicoside.

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