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Determination of total cysteamine in human serum by a high-performance liquid chromatography with fluorescence detection

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Abstract

A convenient, reliable and rapid method for the determination of total cysteamine in human plasma by high-performance liquid chromatography with fluorescence detection is reported. This assay involves reduction of samples with dithiothreitol, derivatization of total cysteamine by addition of monobromobimane and protein precipitation by perchloric acid. The calibration curve was linear in the range 2–150 nmol ml^{-1} and the detection limit was 0.5 nmol ml^{-1}. This method was successfully applied for a pharmacokinetic study of three cysteamine derivatives in healthy volunteers without any interference from coexisting substances.

Keywords: Clinical trial; Cysteamine; Phosphocysteamine; High performance liquid chromatography; Fluorimetric detection; Monobromobimane; Bioavailability

1. Introduction

Nephropathic cystinosis is a rare autosomal recessive disease characterised by an excessive intra lysosomal cystine accumulation due to a defect of its transport system in the lysosomal membrane [1]. This metabolic trouble leads to a multisystemic disease affecting all organ systems. Thus, within the first years of life, new born children develop a renal Fanconi syndrome with polyuria, glucosuria, phosphaturia and aminoaciduria. During their first decade of life, children are affected by growth impairment and progressive decline in renal function leading to hemodialysis or renal transplantation. In affected children, cystine accumulation is also responsible for delayed complications such as hypothyroidism, retinopathy, myopathy, pancreatic dysfunction and dementia [2].

First, in vitro experiment highlighted the cystine-depleting effects of cystamine on cystinotic fibroblasts [3]. These results were confirmed on leukocytes in vivo [3]. The underlying mechanism

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of action was shown to be a disulphide exchange reaction between cysteamine and cystine. The product of the reaction, a mixed disulphide of cysteamine and cystine, is carried out of the lysosome by a lysine porter which is not defective in cystinosis patients [1,2].

Based on this biochemical evidence, cysteamine appears to be a promising treatment for cystinosis. Several long term clinical trials have shown that cysteamine administration (as cysteamine hydrochloride) stabilises renal function, delays glomerular deterioration and improves linear growth [1]. Nevertheless, this drug seems to have different disadvantages: a bad taste and smell that make compliance difficult for young patients, low bioavailability ($< 10\%$) and administration in divided doses (three or four daily). Therefore, in order to overcome these disadvantages, a prodrug, phosphocysteamine, as effective as cysteamine according to its cysteine depleting properties in equimolar doses, has been developed [4]. With respect to its longer lasting effects on cysteine depletion, some authors reported two administrations per day being of major benefit to the patients [2]. Furthermore, a benefit was shown regarding taste preferences [4]. In 1994, another salt of cysteamine, cysteamine bitartrate (CYSTAGON®), was approved by the US Food and Drug Administration for cystinosis treatment [5].

Schneider has shown greater patient compliance in taking CYSTAGON® capsules than the liquid forms of cysteamine hydrochloride or phosphocysteamine. He has also emphasised the fact that both drugs are equally effective in maintaining glomerular function in cystinosis patients [5].

Despite numerous clinical trials, many questions still remain concerning cystinosis systemic therapy. Orphan drug evaluation is limited by the small number of patients, preventing firm conclusions with respect to recommended doses and clinical effectiveness of these medications. Therefore, for the first time to our knowledge, a clinical trial has been proposed in order to estimate phosphocysteamine and cysteamine bitartrate bioavailability in comparison to cysteamine hydrochloride and their pharmacokinetic profiles prior to dose-effect studies.

As no difference could be expected in bioavailability between adults and children, as well as for ethical reasons, this study was conduced on healthy adults. This random double-blind cross over study was designed with a 3 day 'wash-out' period between each drug. Eighteen healthy volunteers received equimolar oral single doses (1200 mg of cysteamine basis) of either: cysteamine hydrochloride, bitartrate or phosphocysteamine. Several blood samples were taken at various times over a 12 h period after each administration in order to monitor total plasma concentration of cysteamine.

With this goal in mind, a rapid but specific and sufficiently sensitive analytical method was required for total cysteamine determination in biological fluids. Moreover, in the field of pharmacokinetic study, the practical nature of an analytical procedure for the treatment of numerous samples (about 800 in our study) and the availability of the equipment in the hospital pharmacy appear to be essential.

In the literature, numerous analytical methods have been reported for the quantitation of thiols and disulphides (cysteine, homocysteine, glutathione) based on a wide variety of detection and separation techniques. These methods include: chemical [6] and enzymatic techniques [7–9], high-performance liquid chromatography (HPLC), gas chromatography, flow cytometry and capillary electrophoresis [10]. Thiols and disulphides can be detected using spectrophotometric [10], spectrofluorimetric or electrochemical detectors [10,11].

Despite the physiological and therapeutic properties of cysteamine, only a few analytical procedures have been published for its determination in biological fluids. These techniques were based on ion-exchange column chromatography [12], high-voltage electrophoresis [13], HPLC methods with fluorimetric [14,15], or electrochemical detection [16,15] and on gas chromatography with flame photometric detection [11].

According to the published data, both ion-exchange column chromatography and electrophoresis lack sensitivity [17]. Therefore, we focused our attention on more sensitive procedures, such as HPLC with fluorimetric or electro-

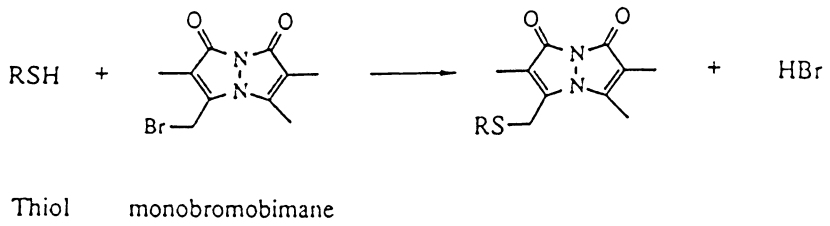

Fig. 1. Reaction of cysteamine with monobromobimane (BBr).

chemical detection and gas chromatographic analysis with flame photometric detection. Based on the suitability and practicability of liquid chromatographic techniques and also on its availability in hospital pharmacies, we chose to use HPLC with fluorimetric detection.

Several derivatization reagents are described in the literature for the fluorimetric detection of cysteamine and thiol derivatives, but these usually require time-consuming pre-treatment of the biological samples. In this context, monobromobimane (BBr) appeared to be a useful thiol derivatization agent, with respect to its specificity for thiols, good separation of fluorescent derivatives of BBr and also the suitability of the sample preparation conditions (reaction at room temperature) [18,19], the direct quantitation after derivatization (without previous extraction) and the stability of the fluorescent derivative [18].

Furthermore, in order to determine reliable storage conditions and quantitation techniques, we focused on the validation of the analytical procedures, as well as on the validation of the sample collection and sample storage conditions.

2. Materials and methods

2.1. Chemicals

Cysteamine hydrochloride and the ion-pairing reagent (octane sulphonate) were purchased from SIGMA. Calf serum was obtained from Bio-Whittaker, methanol (HPLC reagent) from JT Baker and sulphuric acid (p.a.) from Merck. Filters (0.22 μm; Millex GS) were supplied by Millipore.

An aqueous solution (100 mM) of the reduction reagent (dithiothreitol from Sigma) was used. The derivatization agent (monobromobimane Thiolyte® from Calbiochem) was prepared in methanol (4 mM), stored at 4°C and protected from light for up to 4 months. The deproteination agent (perchloric acid pro analysis from Fluka) was diluted in water (0.25 M).

2.2. Equipment

The HPLC device consisted of an isocratic pump (Shimadzu RF-530; Kyoto, Japan), an automatic injector and autosampler (Marathon), a fluorescence HPLC monitor (RF-551 Touzart and Matignon) and an integrator (Shimadzu C-R4AX Chromatopac). The excitation and emission wavelenghs were set at 350 and 480 nm, respectively.

2.3. Procedure

Standard cysteamine solutions: cysteamine hydrochloride was disolved in deionised water (0.62 mM) and used to spike human drug free serum in order to obtain the stock standard solutions (10, 30, 60 and 100 μM of cysteamine). Aliquots of these solutions were stored at −20°C for less than 4 months.

2.3.1. Sample collection and treatment

Venous blood samples from normal volunteers were taken and kept in ethylenediamine tetraacetate (Vacutainer Hemogard EDTA 5 ml, Becton Dickinson, France). Plasma and red cells were immediately separated by centrifugation. The plasma layer was carefully collected and the plasma samples were stored at −20°C for up to 4 months without any significant change in their cysteamine content. These results confirmed that delayed analysis of the biological samples was possible.

2.3.2. Reduction and derivatization procedures

In order to obtain total plasma cysteamine, both 0.3 ml of stock standard solution and real plasma sample were chemically reduced with 20 μl of dithiothreitol (100 mM) for 15 min at room temperature. To perform derivatization, 0.3 ml of the methanolic solution of BBr were subsequently added. After incubation in the dark at room temperature for 15 min, the mixtures were deproteinised by the addition of an equal volume of perchloric acid (0.25 M). The samples were then centrifuged for 15 min at 3000 rpm and the supernatants were filtered through 0.22 μm filters prior to HPLC analysis.

2.3.3. Chromatography

HPLC separation was carried out under isocratic conditions using a Hypersil ODS reversed phase column (L 100×2.1 mm id, 5 μm; Hewlett–Packard) protected by a precolumn: Guard column RP C18 (Interchim, Ref: 10–02–00007, Opti-guard). The mobile phase consisted of water and methanol (65:35 V/V) spiked with 0.15 g per 100 ml of octane sulphonate. The pH of the mobile phase was then adjusted to 2.8 with sulphuric acid. An injection volume of 20 μl was used. At a flow rate of 0.3 ml min^{-1}, cysteamine eluted at about 11 min.

3. Results and discussion

3.1. Reduction of disulphides

The quantitation of cysteamine in human plasma was conducted on aliquots stored at $+4$ or -20°C for several durations: 1, 2, 3 and 24 h

Table 1
Within and between-day variabilities

	RSD %	
Level (μ mol l^{-1})	Within-day ($n=10$)	Between-day ($n=10$)
2	10.4	11.5
60	1.2	2.2
150	0.7	0.8

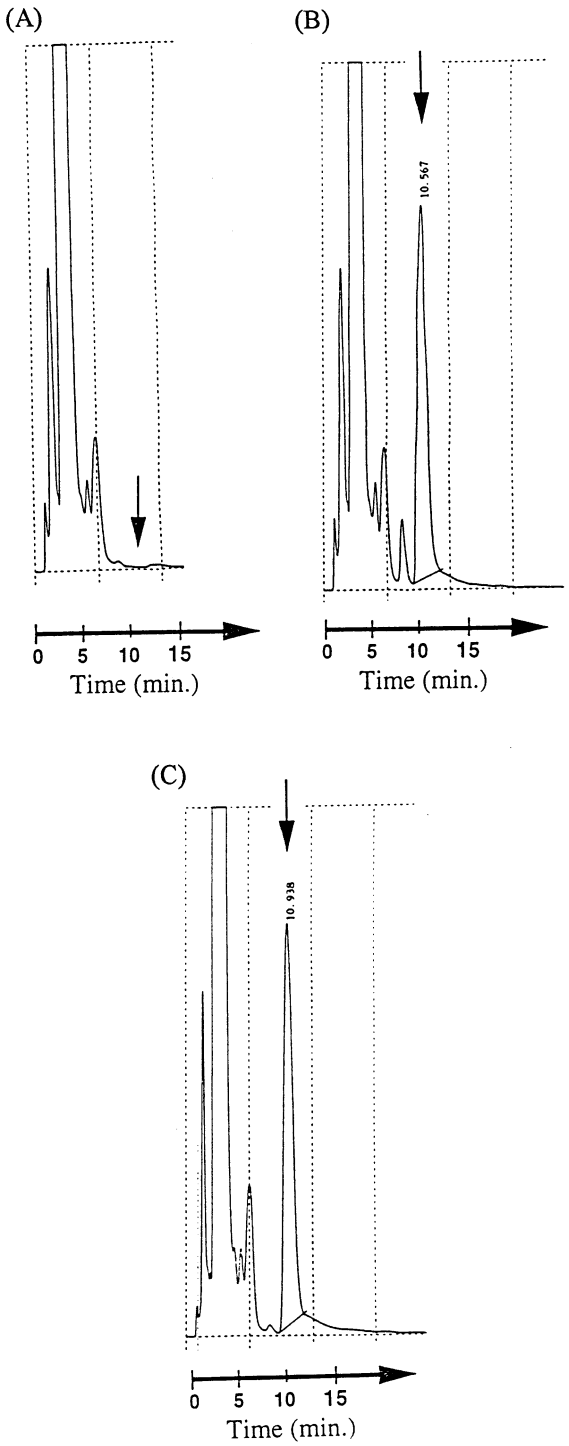

Fig. 2. Typical chromatograms obtained from: (A) human drug free plasma; (B) human plasma spiked with 60 μmol l^{-1} of cysteamine hydrochloride; and (C) from human plasma 2 h after administration of the equivalent of 1200 mg of cysteamine basis.

and 7 days. Under these conditions, a significant and very rapid decrease of cysteamine was observed. Only 45% of the original amount was detectable after 1 h and only 10% after a 24 h incubation.

This well-known feature is due to cysteamine oxidation leading to the formation of mixed disulphides with low molecular weight thiols or proteins (Fig. 1). Therefore, we focused our attention on the conditions of reduction of cysteamine required to break the disulphide links and convert them to their thiol form. The addition of dithiothreitol (100 μM) and the incubation for 15 min appeared to provide optimal conditions. Consequently, the measurement obtained represented the total amount of cysteamine in both its reduced (free) and disulphide form.

Under these conditions, after 5, 7 and 35 days and 4 months, the cysteamine recovery was 100 ± 2% of the initial concentration. Furthermore, we observed that the nature of the container (plastic or glass tubes) has no influence on the recovery of cysteamine hydrochloride. Hence, the preparation of the standards and samples were performed in plastic tubes for safety and cost purposes.

Standard solutions of cysteamine were assayed in two different biological media: human and calf serum. Recovery was found to be 100.1 ± 2.6% in calf serum compared to human serum.

Standards were prepared in human serum, but calf serum could also be employed, as no significant difference could be shown between standards prepared in these two media. These results imply quantitative derivatization of total cysteamine with BBr after reduction, providing evidence that the deproteination method also quantitatively releases cysteamine from plasma proteins.

After thawing at room temperature, the reduction of cysteamine was performed extemporaneously prior to derivatization. Under these conditions, a good recovery of total cysteamine in biological samples stored at −20°C over a 4 month period was found. If a second defrosting of the samples at room temperature or 37°C is required (i.e. for reanalysis), we verified that no cysteamine alteration occurred. These results allowed delayed analysis, which is useful in the field of pharmacokinetic studies.

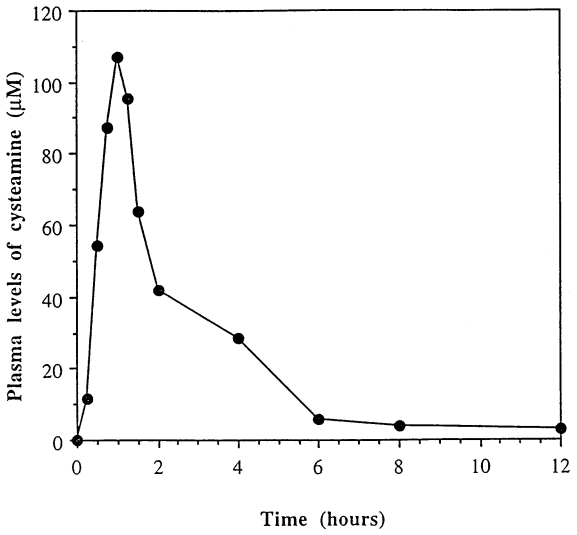

Fig. 3. Plot of plasma total cysteamine concentration versus time after an oral dose of 1200 mg of cysteamine basis.

3.2. Optimisation of derivatization and deproteination

It has been shown that derivatization of thiol with BBr occurs under alkaline conditions and that the reaction products remain stable under acidic conditions [20]. Derivatization by BBr was conducted after reduction of the disulphides. The pH adjustment required for the action of BBr was performed during the reduction of disulphide derivatives by dithiothreitol. Following this, the addition of perchloric acid stopped the derivatization reaction and acted as an effective deproteination agent. Thus, the pH adjustment after derivatization and the deproteination were performed simultaneously in one step.

In contrast, some authors [19] have shown that derivatization and protein precipitation can be achieved simultaneously using methanolic BBr. Nevertheless, under our experimental conditions, the partial deproteination has lead us to add a second step (by perchloric acid) to complete the process. This addition also produced the acidic conditions ($pH < 4$) required to stabilise the fluorescent derivatives of cysteamine.

Thus, such a procedure appeared to be more suitable and rapid than the method described in Ref. [19]. The incubation is performed quickly at room temperature instead of by incubation at +4°C and at ambient temperature for about 1 h, and only one centrifugation of 15 min is required (versus 20 min twice for Yang et al. [19]). Furthermore, under clinical trial conditions, it is very difficult to treat biological samples extemporaneously as described in Yang's procedure [19]. This method required rapid treatment of the biological samples (deproteination and derivatization) before freezing for delayed analysis.

After the deproteination step, a filtration step was required; we have shown that the filtration devices are important. It was observed that some devices (Sartorius Minisart® Ref. 16555; GHP Acrodisc 13, Gelman Sciences) produce interferences in the chromatographic separation of cysteamine. This feature could be due to the membrane material (polypropylene for Acrodisc devices) or to the membrane treatment (cellulose acetate for Sartorius devices). In this case, even though a preliminary wash of the devices overcame this phenomenon, we found Millex GS filters from Millipore (cellulose nitrate and acetate membrane) did not interfere with our separation technique, without any pretreatment.

3.3. Derivative stability

Peak areas for the total derivatised cysteamine (reaction mixture kept at room temperature or in a refrigerator at +4°C) were monitored for at least 7 days. No significant changes were observed for 18 h at ambient temperature in darkness, nor for 7 days at +4°C. The cysteamine derivative is sufficiently stable to allow automatic chromatographic analysis, which is required due to the large quantity of biological samples collected for pharmacokinetic studies.

3.4. Analytical characteristics of merit

The assay was validated by addition of cysteamine hydrochloride to human plasma samples. Cysteamine hydrochloride was chosen as the standard raw material for generating calibration curves for its quantitation, but also for the quantitation of bitartrate and phosphocysteamine. Cysteamine bitartrate and phosphocysteamine are rapidly metabolised into cysteamine hydrochloride, which is the active circulating form of cysteamine.

The analytical method is highly specific, and cysteamine could be detected without any interference from coexisting substances, as shown on the chromatograms. The calibration curve, peak area versus cysteamine concentration in plasma, was linear over the investigated concentration range (2–150 μmol l^{-1}) with a correlation coefficient of 0.999.

The within-day and between-day variabilities (expressed in RDS %) for cysteamine determination in plasma samples spiked at several analyte levels are reported in Table 1. The limit of quantitation was 2 nmol ml^{-1}, which is adequate for monitoring the 12 h time-course of cysteamine concentration in plasma of subjects under treatment. A detection limit (signal-to-noise ratio of 3) of 0.5 nmol ml^{-1} was obtained.

3.5. Preliminary results on human volunteers

The developed method was successfully applied for the pharmacokinetic study in the context of clinical trials. Initial results have shown that no cysteamine was found in the plasma of the healthy volunteers prior drug administration (baseline at $t = 0$), confirming the high specificity of the analytical procedure (Fig. 2).

As shown in Fig. 3, the method appeared to be sufficiently sensitive, as the quantitation limit was not reached after 12 h. Furthermore, pharmacokinetic parameters were calculated with good precision for all subjects.

In conclusion, we have developed a reliable and convenient method for the rapid and accurate determination of plasma total cysteamine levels. It appears to be a suitable tool for pharmacokinetic studies in hospitals.

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